

Use of Bowman Birk Inhibitor for the Treatment of Multiple Sclerosis  
and Other Autoimmune Diseases

## REFERENCE TO RELATED APPLICATION

- 5           This application claims priority to US Provisional Application 60/230568, filed September 2, 2000.

## FIELD OF THE INVENTION

- 10           This invention relates generally to novel use of the Bowman-Birk protease inhibitor for the treatment of neuroinflammatory autoimmune diseases, such as Multiple Sclerosis and Guillain Barre Syndrome, and other inflammatory autoimmune diseases, such as rheumatoid arthritis.

## BACKGROUND OF THE INVENTION

- 15           The Bowman-Birk protease inhibitor (BBI) was first described decades ago (Bowman *et al.*, *Arch. Biochem. Biophys.* 16:109-113 (1948); Bowman *et al.*, *Proc. Soc. Exp. Biol. Med.* 57:139-140 (1944)). The BBI protein consists of 71 amino acid residues and 7 disulfide bonds, and it has a molecular weight of 7975 daltons (Odani *et al.*, *J. Biochem.* 74:697-715 (1973)). BBI contains two functional protease inhibitor domains of different specificities. It inhibits  
20 both trypsin and chymotrypsin-like proteases (Birk *et al.*, *J. Peptide Protein Res.* 25:113-134 (1985)), wherein one domain inhibits chymotrypsin-like proteases, and the other inhibits trypsin-like proteases. Chymase and tryptase are serine proteases, which are stored in the cytosol, from which they may be released upon stimulation by potentially pro-inflammatory cells, such as mast cells or macrophages.

- 25           The potent ability of certain serine protease inhibitors, such as BBI, to prevent the malignant transformation of cells was discovered in the laboratory of Dr. Ann Kennedy more than two decades ago (*e.g.*, US Patent Nos. 5,217,717 and 5,338,547; Kennedy *et al.*, *Nature* 26:825-826 (1978); Yavelow *et al.*, *Proc. Nat'l. Acad. Sci. USA* 82:5395-5399 (1985); Kennedy, In Protease-Inhibitors as Cancer Chemopreventive Agents, Troll, W, Kennedy, AR  
30 (eds), New York, Plenum Press, 1993A, pp. 9-64; Kennedy, *Pharmacological Therapeutics* 78:167-209 (1998)).



Bowman Birk Inhibitor Concentrate (BBIC) is a soybean-derived extract enriched in the protease inhibitor, BBI, developed by Dr. Kennedy as a cancer chemopreventative agent (Kennedy *et al.*, *Nutr. Cancer* 19:281-302 (1993B); US Patent Nos. 4,793,996; 5,217,717 and 5,338,547). The use of BBIC is preferred over crude soybean extract because: a) a very large amount of crude soybean extract would be required to contain amounts of BBI equivalent to the proposed dose of BBIC (approximately 2 quarts of soybean milk or the equivalent amount of tofu); b) crude soybean extract may also contain components which actually counter some of the anticipated beneficial effects of BBI.

Because the anti-carcinogenic activity of BBI is associated with the chymotrypsin-inhibitory domain of BBI (Yavelow *et al.*, 1985), BBIC is quantitated in chymotrypsin inhibitor (CI) units (one CI unit is defined as the amount required to inhibit one mg of chymotrypsin (Kennedy *et al.*, 1993B). In April 1992, BBIC was granted Investigational New Drug status (IND # 34671) from the Food and Drug Administration (FDA), and human trials to evaluate BBIC in several disease states are completed or in progress.

It is clear from animal studies that orally ingested BBI is absorbed and has systemic effects (reviewed in Kennedy, 1998). The structure of the BBI molecule is extremely stable (Birk, 1985; Birk, *Meth. Enzymol.* 45:695-751 (1975)), such that it survives the digestive process as an intact protease inhibitor capable of inhibiting proteolytic activities. This contrasts with a number of other protease inhibitors which do not survive the digestive process (Kennedy, unpublished). Approximately 50% of the ingested BBI is absorbed into the bloodstream.

BBIC is reportedly a better inhibitor of human chymases than any physiologic protease inhibitor described to date. In a recently finished Phase IIa oral cancer chemoprevention trial in patients with pre-malignant lesions known as oral leukoplakia, daily doses of BBIC led to a significant decrease in lesion size in a dose-dependent manner (Meyskens *et al.*, *Proc. Amer. Assoc. Cancer Res.* 40:Abstract #2855 (1999).

Significantly, no toxicity has been observed due to BBIC in any human trial (see Table in Detailed Description). No antibodies against BBIC have been found in the sera of any patients receiving BBIC orally (Maki *et al.*, *Nutr. Cancer* 22:185-193 (1994); Kennedy, personal communication; US Patent No. 5,961,980).



Absorbed BBI is measurable using antibodies to reduced BBI, produced by injection into experimental animals and utilized in immunoassays (Wan *et al.*, 1995). BBI has been assessed in the blood, tissue and urine of rodents and dogs after the ingestion of BBIC permitting pharmacokinetic studies, although it has not yet been feasible to measure BBI levels in the blood of humans after oral BBIC dosing. However, it has been found in the urine, starting within several hours after a single oral dose (Wan *et al.*, *Cancer Epidem. Biomarkers & Prevention* 9:741-747 (2000)). Of note, studies in orally-dosed animals have shown that some BBI can be subsequently found in the CNS even when the blood-brain barrier is intact (Kennedy, AR, personal communication).

BBIC has also proven in many instances to be an effective anti-inflammatory agent. BBIC has been demonstrated to have a suppressive effect on inflammation occurring in carcinogen-treated rodents, as measured by the level of inflammatory infiltrates or lymphoid aggregates, in organs such as the colon and esophagus. For example, in the treatment of ulcerative colitis, an inflammatory bowel disease (Ware *et al.*, *Digestive Diseases and Sciences* 44:896-90 (1999)), inflammation was significantly reduced following treatment with BBIC. Moreover, the chemical induction of ulcerative colitis in rats resulted in the induction of many proteolytic activities in the lesioned, inflamed tissues, on which BBI/BBIC reportedly showed a highly significant inhibitory effect on essentially all of the induced proteolytic activities (Hawkins *et al.*, *Digestive Diseases and Sciences* 42:1969-1980 (1996)). In addition, BBI/BBIC treatment resulted in a suppression of cancer development and a reduction in the levels of inflammation, as measured by the level of inflammatory infiltrates or lymphoid aggregates in the colon (Kennedy *et al.*, 1993A).

Several possible mechanisms by which BBIC may cause these effects have been proposed. First, it has been suggested that BBIC interferes with the inflammatory response by reducing the production of oxygen radicals in polymorphonuclear leukocytes. Second, BBIC reportedly decreases interleukin-1 (IL-1) release, which is a well known, pro-inflammatory cytokine, participating in a wide variety of immune and inflammatory reactions. Third, BBIC has been shown to have the ability to inhibit the malignant transformation of cells; it has been hypothesized that BBI may inhibit cell transformation by affecting the function of certain oncogenes/proto-oncogenes (*e.g.*, c-myc and c-fos). Nevertheless, little is known yet about



either the cellular or molecular mechanisms by which BBIC can modulate or ameliorate autoimmune diseases.

BBI has been shown to efficiently inhibit several identified proteases released from human inflammation-mediating cells. These include human leukocyte elastase Tikhonova *et al.*, *Biochemistry* (Moscow) 59:1295-1299 (1994); Larionova *et al.*, *Biochemistry* (Moscow) 58:1437-44 (1993)) and human cathepsin G (Larionova *et al.*, 1993; Gladysheva *et al.*, *Biochemistry* (Moscow) 59(4):513-518 (1994)), which can efficiently destroy matrix molecules and severely damage tissues.

It is also known that BBI, as well as several other inhibitors of chymotrypsin proteolytic activity, have the ability to prevent the induction of superoxide anion radicals and hydrogen peroxide from stimulated human polymorphonuclear leukocytes and macrophage-like cells (Frenkel *et al.*, *Carcinogenesis* 8:1207-1212 (1987); Ware *et al.*, *Cancer and Nutrition* 33:174-177 (1999)). Proteases and free radicals produced by macrophages are closely associated with the production of inflammation. For example, Multiple Sclerosis (MS) is characterized by inflammation and increased numbers of activated immunocytes of macrophage and T cell lineage (Hauser *et al.*, In Harrison's Principles of Internal Medicine. Fauci *et al.* (eds). New York, McGraw-Hill, 1998, pp. 2409-2419).

#### *Multiple Sclerosis (MS).*

Multiple sclerosis (MS) is a neuroinflammatory disease of the central nervous system (CNS) characterized by chronic inflammation, demyelination and gliosis. Pathologically, MS is characterized by well-demarcated, macroscopic lesions, called plaques, in the brain white matter and, less frequently, gray matter. Acute lesions are characterized by perivenular cuffing and infiltration of T lymphocytes and macrophages, along with a few B cells and plasma cells. MS is reportedly an autoimmune disorder, likely triggered by environmental exposure in a genetically susceptible host. Complications from MS may affect multiple physiological systems and require profound changes in lifestyle for patients and their families. MS affects 350,000 Americans and is the second most frequent cause (after trauma) of neurologic disability in early to middle adulthood (Hauser *et al.*, 1998).

MS is a complex disease, manifested in progressive or relapsing modalities, or combinations thereof. Proteolysis of myelin appears key to demyelination, which, in association with perivenular inflammation, is a major pathological feature in multiple sclerosis.



Much of the investigative interest in MS has focused on the possible toxic effects on CNS myelin of locally accumulated lymphocytes and their components/products (Bar-Or *et al.*, *J. Neuroimmunol.* 100 :252-259 (1999); Wucherpfennig *et al.*, *J. Clin. Invest.* 100(5):1114-1122 (1997).

5 Proteases are associated with many facets of immune system function and immune system disorders (Cuzner *et al.*, *J. Neuroimmunol.* 94:1-14 (1999); Vaday *et al.*, *J. Leukoc. Biol.* 67:149-159 (2000)). A variety of proteases are increased in MS lesions, including lysosomal proteases and matrix metalloproteinases gelatinase A and B (MMP-2 and 9, respectively) (Cuzner *et al.*, 1999; Halonen *et al.*, *J. Neurol. Sci.* 79:267-274 (1987); Kieseier *et al.*, *Curr. Opin. Neurol.* 12:323-336 (1999); Hartung *et al.*, *J. Neuroimmunol.* 107:140-147 (2000); Bever *et al.*, *Neurology* 53:1380-1381 (1999); Maeda *et al.*, *J. Neuropathol. Experimental Neurol.* 55:300-309 (1996)).

15 Macrophages are also observed in association with chronic MS plaques (Hauser *et al.*, 1998), even in early plaques, raising the possibility the macrophages may release myelinotoxic agents as well as serving a scavenger role (Cuzner *et al.*, 1999; Raine, *Ann. Neurol.* 36:S61-72 (1994)). Macrophages are known to release MMPs, which are characteristically released from cells in the form of an inactive proenzyme, which must be activated by proteolytic removal of a propeptide (Atkinson *et al.*, *Biochem. J.* 288:605-611 (1992); Cuzner *et al.*, 1999; Hartung *et al.*, 2000). This activation step can be carried out by other MMPs (Atkinson *et al.*, 1992), or by  
20 serine proteases, such as plasmin, cathepsin G, chymase and trypsin (Cuzner *et al.*, 1999; Hartung *et al.*, 2000; Murphy *et al.*, *Ann. N.Y. Acad. Sci.* 667:1-12 (1992); Brosnan *et al.*, *Nature* 285:235-237 (1980)).

Mast cells also frequently accumulate in the cellular areas of MS plaques (Cuzner *et al.*, 1999; Ibrahim *et al.*, *J. Neuroimmunol.* 70:131-138 (1996)). Therefore, it is of interest that  
25 mast cells reportedly contain two serine proteases (cathepsin G [ $K_i=1.2$  nM] and chymase [ $K_i=50$  pM]), which are released with histamine upon degranulation. Active at neutral pH, the serine proteases may not only play an important initial role in the activation of MMPs in the pro-inflammatory enzymatic cascade, but they could account for the cleavage of the myelin components and release of the encephalitogenic fragments (Opdenakker *et al.*, *Immunol. Today*  
30 15:103-107 (1994)). Taken together, these observations indicate that mast cell proteases play a role in MS, and suggest that inhibition of serine protease activity is a potentially important



therapeutic approach in MS, particularly if the production of naturally occurring anti-proteinases is impaired in MS lesional areas. Currently, BBI is the best known natural inhibitor of mast cell chymase (Ware *et al.*, *Arch. Biochem. Biophys.* 344:133-138 (1997)).

Agents presently used prophylactically against MS relapses include interferon  $\beta$ 1a,  $\beta$ 1b, and copolymer-1. These agents are administered by subcutaneous or intramuscular injection on a daily or every other day basis. While generally well tolerated, the benefit of these therapies is limited to reducing the MS relapse rate by only about one third, when compared to placebo recipients. Moreover, neutralizing antibodies against interferon are produced within 12 months of initial treatment by significant numbers (20-40%) of those patients receiving current therapies, causing those patients to return to their pretreatment relapse rate. Acute relapses may be treated with a brief course of intravenous methylprednisolone, followed by oral prednisone. However, such short-term glucocorticoid therapy is associated with fluid retention, weight gain, gastric disturbances and emotional lability, which may require further treatment.

Chronically progressing MS is sometimes treated with immunosuppressants, such as methotrexate, azathioprine or cladribine. However, while these agents are of modest efficacy, regular monitoring of a patient's blood cell counts and liver functions during therapy is advised due to potential toxicities. Spasticity can be treated with agents, such as baclofen, diazepam, or clonazepam, but these are all of limited efficacy and can be counterproductive for patients who require a degree of rigidity for daily activities, such as walking.

#### *Experimental autoimmune encephalomyelitis (EAE).*

Experimental autoimmune encephalomyelitis (EAE) is a long-established disease model for MS. First described in monkeys (Rivers *et al.*, *J. Exp. Med.* 58:39-53 (1933)), this paradigm has been reproduced in several species, including mice and rats. EAE is induced by immunizing with myelin components, purified myelin proteins, or by peptide fragments resulting from the cleavage of stable encephalitogenic peptides from myelin, using a protease released from degranulated mast cells at neutral pH (Dietsch *et al.*, *Cell. Immunol.* 135:541-548 (1991); Constantinescu *et al.*, *Immunologic Res.* 17:217-227 (1998); Constantinescu *et al.*, *J. Immunol.* 161:5097-5104 (1998)). EAE can also be induced 'passively' by adoptive transfer of antigen-reactive T helper cells from an immunized animal.

Histopathologically, EAE is characterized by CNS inflammation with macrophage and lymphocytic infiltrates and varying degrees of demyelination (Raine, In Textbook of



Neuropathology, Davis RL (ed) Baltimore, MD, Williams and Wilkins, 1990, pp. 356-358).

The disease manifests clinically with paralysis, beginning at the tail and spreading rostrally to the hindlimbs and forelimbs, and in advanced stages affects breathing and causes death.

*Experimental Autoimmune Neuritis (EAN).*

- 5 Oral BBIC administration has also demonstrated a profound inhibitory effect in rats with experimental autoimmune neuritis (EAN), an animal model of autoimmune peripheral nerve demyelinating disease having clinical, pathological and electrophysiological similarities to human Guillain-Barre Syndrome (GBS). GBS is an autoimmune, neuroinflammatory disorder related to MS, but affecting primarily the peripheral nervous system (PNS) (Shang *et al.*, *J Immunol.* 160:467 (1998); Owens *et al.*, *Immunol. Today* 15:566 (1994)).

- 10 EAN is a T cell mediated disease that can be transferred by CD4-positive antigen-specific Th<sub>1</sub> cells. Histopathologically, EAN is characterized by T cell and macrophage infiltration of the nerve roots (Weber *et al.*, *J. Cell. Biol.* 134:1063 (1996)), demyelination and axonal injury (Waksman *et al.*, *J. Exp. Med.* 102:213-236 (1955); Rosen *et al.*, *Muscle and*  
 15 *Nerve* 13:629-636 (1990); Vaddi *et al.*, *J. Immunol.* 153:4721 (1994)). The infiltration of cells to the target tissue is accompanied with increased expression of adhesion molecules, pro-inflammatory cytokines and chemokines, both by the infiltrating cells and at the site of the immunological insult. Clinically, EAN manifests in rats first with tail paralysis, and progresses rostrally with paralysis of the hindlimbs, then the forelimbs. However, when model animals  
 20 were treated with BBIC, a reduction was seen in both the extent of demyelination and the accumulation of inflammatory cells in the PNS tissue, comparable to the effect in EAE.

- EAN is induced in rats by the injection of whole peripheral nerve tissues (Waksman *et al.*, 1955; Rostami, *Springer Semin. Immunopathol.* 17:29-42 (1995)), a protein fraction (P0 or P2) isolated from peripheral nerve myelin (Brostoff *et al.*, *Nat. New Biol.* 235:210-212 (1972);  
 25 Rostami *et al.*, *Ann. Neurol.* 16:680-685 (1984)), or by synthetic peptides corresponding to the myelin proteins (peptide SP26 corresponds to the 53-78 amino-acid sequence of the myelin P2 protein) (Rostami *et al.*, *J. Neuroimmunol.* 30:145-151 (1990)).

- Inflammatory autoimmune diseases, such as rheumatoid arthritis, and neuro-inflammatory autoimmune diseases, such as Multiple Sclerosis (MS) and Guillain Barre  
 30 Syndrome, exert a major impact on the health of the American population. MS is particularly devastating because of the extensive morbidity and premature fatalities in relatively young,



productive individuals afflicted with the disease. Current therapeutic approaches to MS, such as treatment with glatiramer or beta-interferon (INF- $\beta$ ) have resulted in only relatively modest benefits. However, the disease is not well controlled. The use of other therapeutic agents, such as corticosteroids and immunosuppressive agents, in recent years has also been limited by inconsistent benefits and/or cumulative toxic effects. Therefore, from both a social and a medical standpoint, there is a major need for a reliable and effective non-toxic method for treating the chronic inflammatory effects that play pathogenic roles in MS, GBS, and other autoimmune diseases.

## 10 SUMMARY OF THE INVENTION

The present invention provides a non-toxic therapy and a novel use for Bowman Birk Inhibitor (BBI), as administered in Bowman Birk Inhibitor Concentrate (BBIC), for the treatment of autoimmune diseases characterized by chronic inflammation in a patient, such as rheumatoid arthritis, and more particularly for the treatment of those diseases that are characterized by chronic neuroinflammation and/or demyelination, such as Multiple Sclerosis (MS) and Guillain Barre Syndrome (GBS). In addition, the present invention provides methods for using BBI/BBIC to reduce, inhibit, suppress or prevent the chronic inflammation in such patients; and more particularly, to reduce, inhibit, suppress or prevent the chronic inflammation and demyelination that occurs when the patient's nerve tissue is affected by the disease. Such neuroinflammation may affect the central nervous system or peripheral nervous system of the patient.

The method is, therefore, provided for the treatment of chronic autoimmune diseases in the patient characterized by inflammation, such as rheumatoid arthritis, and more particularly for the treatment those diseases that are characterized by neuroinflammation and/or demyelination, such as Multiple Sclerosis (MS) and Guillain Barre Syndrome (GBS).

In a preferred embodiment of the invention, the method of using BBI/BBIC to treat a disease is applied to a human patient, although other animals, particularly mammals may also be treated.

In another preferred embodiment of the invention, the method is provided in which the Bowman Birk Inhibitor is administered orally. In certain preferred embodiments, BBI/BBIC is



administered to the patient with a carrier, or it is administered with another therapeutic agent, drug, medicament or treatment

In yet another preferred embodiment of the invention the method is provided in which the Bowman Birk Inhibitor (BBI) is administered as Bowman Birk Inhibitor Concentrate (BBIC). The BBI is provided as an enriched concentrate extracted from a legume, preferably as an enriched concentrate extracted from soybeans (BBIC).

Also provided in the present invention is a method for treating inflammation in an animal model of an induced inflammatory disease comprising administering to the animal an amount of Bowman Birk Inhibitor effective to reduce, inhibit, suppress or prevent the chronic inflammation. As above, the method is preferably applied when the chronic inflammation is inflammation of neural tissue, as part of either the central nervous system or the peripheral nervous system of the patient. The present method is particularly useful when used to reduce, inhibit, suppress or prevent demyelination of the nerve tissue of the patient. In a preferred embodiment of the present method, the induced disease is Experimental Autoimmune Encephalomyelitis (EAE) or Experimental Autoimmune Neuritis (EAN).

Not only does the use of BBIC, alone or in concert with other agents or treatments, provide a major step forward in the treatment of MS and other chronic inflammatory autoimmune diseases, including neuroinflammatory diseases, its use will permit further studies into the nature of the inflammatory reactions involved in such diseases. Thus, the invention further provides for the use of BBI/BBIC *in vitro* with cells from patients or animal subjects affected by or subject to induced inflammatory responses, such as EAE or EAN, as well as chronic inflammatory autoimmune diseases, such as rheumatoid arthritis, or chronic neuroinflammatory autoimmune diseases, such as MS and GBS.

Additional objects, advantages and novel features of the invention will be set forth in part in the description, examples and figures which follow, and in part will become apparent to those skilled in the art on examination of the following, or may be learned by practice of the invention.

## DESCRIPTION OF THE DRAWINGS

The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawings. For the



purpose of illustrating the invention, there are shown in the drawings, certain embodiment(s) which are presently preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities shown.

FIG. 1 graphically depicts the effect of treatment of active EAN with BBIC. The clinical score is shown. The data are shown as mean clinical score at any particular time point (n=6 for each group at each time point; \* p< 0.05; compared to untreated animals).

FIG. 2 graphically depicts the effect of pretreatment with BBIC to ameliorate the clinical course of passive EAN. Disease severity was scored according to the above-described scale (mean±SD; n=6 for each group at each time point). The representative experiment of six separate experiments is shown.

FIG. 3 graphically depicts BBIC inhibition of antigen-specific proliferation. Splenocytes, isolated from EAN animals and incubated with or without SP26 in the presence or absence of BBIC, were pulsed with <sup>3</sup>H-thymidine and the <sup>3</sup>H-thymidine incorporation is shown. Values were significantly different (p<0.05) from proliferation values of cells untreated by SP26, and significantly different (p<0.05) from splenocytes stimulated with SP26. The data are representative of ten (10) experiments. Mean ±SD; n=6 is shown.

FIGs. 4A-4L graphically depict a series of FACS analyses of CD3, CD4, and CD8 expression following BBIC treatment *in vitro*. FIGs. 4A, 4E, and 4I, side light scatter (SSC) plots and gates, depict isotype controls. EAN splenocytes were stained and analyzed by FACS for the expression of CD3 (FIGs. 4B-4D), CD4 (FIGs. 4F-4H), and CD8 (FIGs. 4J-4L). The numbers refer to the mean fluorescence intensity (MFI) and average number of positive cells from eight (8) different experiments.

FIGs. 5A-5H graphically depict the increase in macrophage population after exposure to BBIC. FIGs. 5A and 5E, side light scatter (SSC) plots and gates, depict isotype controls. EAN splenocytes were stained and analyzed by FACS for the expression of CD11b (FIGs. 5B-5D) and ED1-like antigen (FIGs. 5F-5H). The numbers refer to the MFI and average number of positive cells from eight (8) different experiments. As shown, BBIC up-regulated expression of ED1-like antigen (FIG. 5H) relative to the expression by either untreated cells (FIG. 5F) or after 72 h incubation with SP26 (FIG. 5G), but BBIC had no effect on CD11b expression (FIG. 5D) compared either with SP26-untreated (FIG. 5C) or -treated cells (FIG. 5B).



FIGs. 6A and 6B graphically depict amplification in response to BBIC treatment on the secretion of IFN $\gamma$  (FIG. 6A), which had no effect on IL-10 production (FIG. 6B) from elicited macrophages isolated from EAN rats in response to SP26. Values were significantly different ( $p < 0.05$ ) from cytokine values of cells untreated by SP26, and significantly different ( $p < 0.05$ ) from macrophages stimulated with SP26. The data are the mean of six (6) experiments  $\pm$ SEM (three replicates per experiment).

FIGs. 7A and 7B graphically depict the regulation of nitrite (NO) release by BBIC. Rat elicited macrophages, isolated from EAN animals and incubated with or without (control) BBIC in the presence or absence of SP26, were assayed for NO production after 4 hours (FIG. 7A) or 24 hours (FIG. 7B). Values were significantly different ( $p < 0.01$ ) from NO values of cells untreated by SP26, and significantly different ( $p < 0.05$ ) from macrophages stimulated with SP26. The data are the mean of four (4) experiments  $\pm$ SEM (three replicates per experiment).

FIGs. 8A-8I graphically depict the differential apoptotic response induced by BBIC *in vitro*. Splenocytes isolated from EAN animals, were selected by the forward (FSC-H) and side (SSC-H) light scatters and gated by size (FIG. 8A). In FIGs. 8B-8E, R1 cells are shown; in FIGs. 8F-8I, R2 cells are shown. After 72 hours in culture, cells were examined for apoptotic response without any treatment (FIGs. 8B and 8F), treatment with SP26 (FIG. 8C and 8G), or BBIC (FIGs. 8D and 8H) alone, and in the presence of a combination of SP26 and BBIC (FIGs. 8E and 8I). The percentage of apoptotic cells is shown, calculated as differences between apoptosis in stained cell populations and negative controls developed for each particular group. The representative experiment of three (3) is shown.

FIG. 9 graphically depicts the clinical effect of BBIC over time in the treatment of Lewis rats with EAE.

FIG. 10 graphically depicts the effect of BBIC for the treatment of Lewis rats with EAE, showing the difference in inflammatory demyelination in the CNS (brain and spinal cord) of BBIC-treated animals as compared with matching, untreated control animals.

## DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

The invention provides a novel method for the use of BBI/BBIC to treat patients with chronic inflammatory autoimmune disease, such as, but limited to, rheumatoid arthritis; and particularly to treat patients with diseases characterized by neuroinflammation and/or



demyelination, such as, but not limited to Multiple Sclerosis (MS) and Guillain Barre Syndrome. The method of using BBI/BBIC effectively reduces, inhibits, suppresses or prevents inflammation in such a patient, and when the disease is characterized by neuroinflammation, the method of using BBI/BBIC effectively reduces, inhibits, suppresses or prevents neuroinflammation and/or demyelination of the nerve tissue of the patient.

In a preferred embodiment of the invention, the method is provided in which BBI/BBIC is used to treat an animal subject in which an inflammatory disease is induced, such as Experimental Autoimmune Encephalomyelitis (EAE) or Experimental Autoimmune Neuritis (EAN). The application of the present method of treating inflammation and neuroinflammation in such an animal subject, as displayed by the alleviation or elimination of paralysis, is also an accepted animal model for the responses found in human patients with MS, GBS, or other chronic inflammatory autoimmune diseases.

The use of BBIC for the treatment of MS according to a preferred embodiment of the present invention was selected for a combination of reasons including, but not necessarily limited to, the following. First, there exists a substantial body of evidence that demonstrates increased local protease deposition in the MS lesions, including the presence of macrophages in the cellular zone of early plaque lesions. This protease activity includes neutral serine proteases, such as plasmin, chymase and cathepsin G. Such serine proteases both have a directly damaging effect on myelin, (Romanic *et al.*, *Brain Pathol.* 4:145-156 (1994)), and they convert metalloproteinase (MMP) pro-enzymes into functionally active enzymes, with potentially toxic effects.

Second, it is not yet known exactly how much BBI enters the human CNS after oral BBIC dosing. However, it is anticipated that BBI entry is increased in the CNS of patients affected by MS because of localized decreases in the blood-CNS barrier in such areas. Also, based on the background evidence reviewed above, one would also expect BBI to inhibit entry of immune/inflammatory cells into the CNS, with potential for reduction of the pathogenic effects of such cells.

Third, in preliminary studies, BBIC has been shown to be a potent inhibitor of several serine proteases. Because neutral serine proteases appear to play an important initial role in the activation of MMPs in the pro-inflammatory enzymatic cascade described above, inhibition of such serine protease activity is potentially important to the therapeutic approach in MS. As



reviewed (Cuzner *et al.*, 1999; Hartung *et al.*, 2000), this is significant because extracellular proteolysis has been associated with several effects of importance in inflammatory demyelination, which although not intended to be limiting, include:

- (1) proteolysis of myelin components, such as myelin basic protein (MBP);
- (2) generation of encephalitogenic peptides which could induce autoimmune reactions;
- (3) proteolysis of basement membranes, leading to increased extravasation of humoral factors and cells (decreased blood-brain barrier), implying an important role for MMP's in the process of T-cell migration into the CNS;
- (4) proteolysis of the extracellular matrix-leading to enhanced inflammatory cell migration after entry into the CNS; and
- (5) proteolysis of cell surface molecules on resident cells leading to cytolysis; and proteolytic activation of other zymogens.

Moreover, the therapeutic modulation of serine protease activity may be particularly important in MS because the inhibition of serine proteases by naturally occurring anti-proteinases may be impaired in MS lesional areas.

These observations are consistent with the notion that mast cell proteases also play a role in MS. If so, BBI, which is a highly effective inhibitor of cathepsin G and chymase, two of the major mast cell proteases, provides an excellent agent of intervention at this point.

BBIC administration has demonstrably inhibited both the clinical and pathologic expression of EAE and EAN, which are the recognized animal models for autoimmune disorders characterized by neuroinflammation and demyelination - MS and Gullain Barre Syndrome. Inhibition of plasmin formation and synthetic inhibitors of MMP activity have both been shown to reduce severity of clinical EAE (Cuzner *et al.*, 1999; Gijbels *et al.*, *J. Clin. Invest.* 94:2177-2182 (1994); Clements *et al.*, *J. Neuroimmunol.* 74:85-94 (1997); Hewson *et al.*, *Inflamm. Res.* 44:345-349 (1995)). MMP inhibitors, including naturally occurring agents such as TIMP, may be expected to work at two levels: 1) by blocking extravasations of inflammatory cells into the CNS, and 2) by inhibiting effector properties of lymphocytes and macrophages in the initial stage of inflammation in the MS lesion, without an apparent inhibitory effect on the sensitization of T cells to myelin components (Cuzner *et al.*, 1999).

Since reactive oxygen species (ROS) can damage both oligodendroglia and myelin in



CNS inflammatory reactions, inhibition of ROS formation by BBI offers another ameliorative effect in MS.

Fourth, in human trials involving patients with oral leukoplakia, benign prostatic hyperplasia, and ulcerative colitis since 1992, BBIC has not caused, or been associated, with any toxic effects or induced immune response when used at doses approximately comparable to those employed in the present invention.

Fifth, BBIC is a reasonably well-defined patented product, classified by the FDA as an Investigational New Drug for use in humans. An adequate supply of BBIC is available for studies of sufficient length to determine: a) the safety of BBIC in MS patients, and b) acquire data relating to therapeutic efficacy.

Consequently BBI/BBIC is a highly effective, non-toxic, anti-inflammatory agent useful for the treatment of MS, as well as for the treatment of EAE, an animal model directly analogous to MS. The response in BBI/BBIC treated rats with EAE is similar to the earlier demonstrated response in BBI/BBIC treated rats with EAN. The method is also highly effective for the treatment of other chronic inflammatory autoimmune diseases, such as rheumatoid arthritis, and chronic neuroinflammatory diseases, such as GBS, although the invention is not to be limited to only the identified examples.

In preferred embodiments of the invention, as set forth by Example, BBI/BBIC produced according to known methods effectively suppresses inflammation, such as that which is found associated with rheumatoid arthritis, and in particular effectively suppresses neuroinflammation of the type characteristically found in autoimmune diseases, including MS and GBS. The clinical study set forth in Example 3 is intended to exemplify the parameters of the method of using BBI/BBIC to reduce, inhibit, suppress or prevent inflammation, preferably neuroinflammation in a patient with MS, GBS or rheumatoid arthritis. However, the parameters set forth therein are intended to be exemplary, not limiting as to the dosage or treatment regime that may be used in accordance with the present methods.

By “reduce,” “inhibit,” “suppress” or “prevent” the chronic inflammation or neuroinflammation is meant to modulate the inflammatory or neuroinflammatory effect of the disease in a patient or animal subject affected by the disease, as compared with a matched, untreated control patient or subject when an effective amount of the BBI/BBIC is administered,



such that the inflammation or neuroinflammation is measurably or visibly changed (*e.g.*, reduced, inhibited, suppressed or prevented, as such terms are commonly understood).

In the present invention, compositions comprising BBI for the treatment of diseases or atypical inflammatory conditions are also provided, particularly for neuroinflammatory conditions involved in autoimmune disease. MS and GBS are, as previously noted, reported to be autoimmune diseases or disorders. Other such autoimmune diseases characterized by inflammation include, but are not limited to, *e.g.*, systemic lupus erythematosus and myasthenia graves. However, in the event that MS or GBS are shown not to be autoimmune in nature, the principles set for herein for their treatment remain unchanged. In a preferred embodiment, the BBI/BBIC compositions further comprise a pharmaceutically acceptable carrier.

By "BBI" it is meant any Bowman-Birk Inhibitor or Bowman-Birk Inhibitor product, including, but not limited to, BBI prepared by methods known in the art and BBI concentrates ("BBIC") prepared in accordance with the method of U.S. Pat. No. 5,217,717. Also provided are methods of treating inflammation, particularly neuroinflammation, in an animal by administering an effective amount of a composition comprising BBI.

By "animal" is meant, but is not limited to, any mammal including humans. In the alternative, the term "patient" is also used to indicate the animal, mammal or human being treated with BBI/BBIC in accordance with the disclosure of the present invention. When animal models are treated by the present method, the term "subject" is more frequently used, rather than "patient."

It is clear from many animal studies that orally ingested BBI is absorbed and has systemic effects (reviewed in Kennedy, 1998). The structure of the BBI molecule is extremely stable, such that it survives the digestive process and appears in the colon, etc., as an intact protease inhibitor capable of inhibiting proteolytic activities (Yavelow *et al.*, *Cancer Res.* 43:2454-2459 (1983); Billings *et al.*, *Canc. Lett.* 62:191-197 (1992)). Many other protease inhibitors which have been studied *in vivo* do not survive the digestive process. Moreover, when BBI reaches the colon in intact, active form after ingestion, approximately half of ingested BBI is taken up into the bloodstream (Billings *et al.*, 1992).

The amount of BBI taken up from the gastrointestinal tract into the blood and distributed to internal organs is such that biological effects from BBI are expected in many



different organ systems. Absorbed BBI is measurable in humans (*e.g.*, US Patent No. 5,961,980).

Antibodies to “reduced BBI” have been produced and utilized to measure blood and urine levels of BBI after the ingestion of BBIC (Wan *et al.*, *J. Immunol. Meth.* 180:117-130 (1995); US Patent Nos. 5,616,492 and 5,618,679). Pharmacokinetic studies utilizing these antibodies have been performed in rodents, dogs and humans (Wan *et al.*, unpublished data). It is also clear that BBI can be internalized by intestinal epithelial cells (Billings *et al.*, *Eur. J. Canc.* 27:903-908 (1991)).

It is further known that dietary concentrations of BBIC/BBI capable of preventing cancer in animals in many different organ systems are not toxic. Yet, these same concentrations of BBI/BBIC are known to affect inflammation in animals (Kennedy, 1993A; Kennedy, 1998; Kennedy, *Preventive Med.* 22:796-811 (1993C); Kennedy, *Cancer Res.* (suppl) 54:1999s-2005s (1994); Kennedy, In Nutrients in Cancer Prevention and Treatment, Prasad (ed) 1995B, pp. 71-82).

Compared to raw soybeans, the trypsin inhibitory activity of BBIC has been greatly reduced and the chymotrypsin inhibitory activity has been increased (Kennedy, 1993B). It was, however, earlier thought that the soybean-derived protease inhibitors had the potential to inhibit the growth of young animals, and, perhaps, contribute to pancreatic cancer development in rats. However, it is now recognized by many investigators that the soybean protease inhibitors are not responsible for the growth suppressing effects of raw soybean products in young animals (Birk, In Protease Inhibitors as Cancer Chemopreventive Agents. Troll W, Kennedy AR (eds). New York, Plenum Press, 1993, pp. 97-106). When BBIC was tested at doses considerably higher than the doses of BBIC typically administered to people, significant drug induced toxicity was not observed in any organ, including the pancreas, in at least three species of animals (mice, rat, hamsters) evaluated in the Kennedy laboratory (*e.g.*, Kennedy, 1993A), and in sub-chronic and chronic toxicity studies performed in animals (rodents, dogs) and studied at Southern Research Institute (SRI) and MPI Research, Inc. (Page *et al.*, unpublished data (Page *et al.*, SRI-CBE-94-135-7482 and SRI-CBE-94-060-7482, Birmingham, AL; Southern Research Institute, 1994; Serota, 560-057 and 560-058, MPI Research, Inc., Mattawan, MI (2000)).



Animal experiments at extremely high doses of BBIC have been carried out for as long as the animals' life spans. Thus, even at extremely high doses of dietary BBIC, no histopathologic alterations in the pancreas have been observed. Consequently, the assumption that the soybean protease inhibitors are involved in rat pancreatic cancer development is  
 5 erroneous, as discussed extensively in, *e.g.*, Kennedy, 1993A-C; Kennedy, 1994; Kennedy, *J. Nutr.* 125:733s-743s (1995C)). In human trials, no adverse effects of BBIC on the pancreas have been observed.

The possibility that soybean protease inhibitors may have impact upon the growth of the rat pancreas is triggered by the ability of the protease inhibitors to inhibit trypsin, but not  
 10 chymotrypsin (Birk, 1985; Birk, 1975), while the ability to inhibit carcinogenesis, and presumably inflammation, is associated with the ability to inhibit chymotrypsin (which is why the strength of BBIC doses is measured in CI units) (Yavelow *et al.*, 1985; Kennedy 1993B). However, the two protease inhibitor sites in BBI are separable and distant from each other in the molecule.

Although presently available in concentrates extracted from soybeans, BBI is also found  
 15 in other members of the legume family of plants, such as adzuki beans, black beans, black-eyed peas, peas, lima beans, kidney beans, navy/white beans, pinto beans, chick peas, peanuts, lentils and the like. Using the methods known in the prior art for preparing the enriched BBIC concentrates from soybeans, one of ordinary skill in the art could readily prepare BBI  
 20 concentrates from other legumes. Although the resulting concentrates may not have the same CI values as soybean BBIC, the key factor is that there is some degree of chymotrypsin inhibition. Thus, the resulting concentrate would be quantifiable in CI units.

On a comparable weight basis, the doses of soybean BBIC needed to prevent cancer development or have an anti-inflammatory effect, are well below the doses of soybean protease  
 25 inhibitor activity associated with triggering the feedback response leading to pancreatic abnormalities (Kennedy, 1993A-C, Kennedy, 1994, Kennedy, 1995C). As a result, even on a theoretical basis, the doses of BBI/BBIC used in the treatment of MS would not create a toxicity problem in human patients.

The only toxicity which has previously been associated with BBI/BBIC treatment of  
 30 animals has been that of causing toxicity to the developing embryo when injected at an extremely high level into pregnant mice (Kennedy, 1993A-C; Kennedy, 1994). At normal



dosages, BBI/BBIC do not have teratogenic effects; in fact, these agents have been shown to prevent birth abnormalities, as has been reviewed by Kennedy, 1993A-C and Kennedy, 1994. No toxicity due to BBIC has been reported in any human trial in which BBIC has been used. Nevertheless, although deleterious effects were not expected from the use of dietary BBI/BBIC, early human clinical trials with BBIC are limited to post-menopausal females so that potential problems for a developing embryo can not occur.

In a dose-ranging trial of BBIC in humans with benign prostatic hyperplasia for a 6 month period, there were found to be no increased adverse symptoms or abnormalities in standard laboratory assays when compared to placebo administration for the same time period (Malkowicz, *et al.*, *The Prostate* 48:16-28, (2001); see also following Tables 1 and 2 listing toxicity study findings). The highest BBIC dose given was 400 CI units twice daily, which is the same as that of the present invention. Accordingly, no toxicity issues are expected in the treatment of humans with BBIC for MS or other inflammatory autoimmune diseases.

Table 1: Toxicities: in Treated Patients

Outcome	Maximum Toxicity Score					
	N	0	1	2	3	4
WBC	14	12 (86%)	2 (14%)	0	0	0
Hemoglobin	14	12 (86%)	2 (14%)	0	0	0
Hematocrit	14	13 (93%)	1 (7%)	0	0	0
Platelet	14	13 (79%)	1 (7%)	0	0	0
Granulocytes a	7	6 (86%),	1 (14%)	0	0	0
Lymphocytes	14	9 (64%)	5 (36%)	0	0	0
Mononuclear Cells ab	6	4 (67%)	2 (33%)	0	0	0
Eosinophils	14	13 (93%)	1 (7%)	0	0	0
Basophils	14	13 (93%)	1 (7%)	0	0	0
Calcium	14	14 (100%)	0	0	0	0
Lipase	14	11 (79%)	2 (14%)	1 (7%)	0	0
Amylase	14	12 (86%)	1 (7%)	1 (7%)	0	0
Sodium	14	10 (71%)	4 (29%)	0	0	0
Potassium	14	12 (86%)	2 (14%)	0	0	0
Chloride	14	11 (79%)	3 (21%)	0	0	0
Carbon Dioxide	14	11 (79%)	3 (21%)	0	0	0
Urea/Nitrogen	14	11 (79%)	3 (21%)	0	0	0
Creatinine	14	12 (86%)	2 (14%)	0	0	0
Total Bilirubin	14	13 (93%)	1 (7%)	0	0	0
ALT	14	10 (71%),	4 (29%)	0	0	0
AST	14	14 (100%)	0	0	0	0
Alkaline Phosphatase	14	14 (100%)	0	0	0	0



- a - These measurements were not taken for Arizona patients.  
 b - One patient had mononuclear cell measurements at baseline only.

Table 2: Toxicities in Control Patients (N=4)

Outcome	Maximum Toxicity Score				
	0	1	2	3	4
WBC	4	0	0	0	0
Hemoglobin	4	0	0	0	0
Hematocrit	4	0	0	0	0
Platelet	3	1	0	0	0
Granulocytes a	2	0	0	0	0
Lymphocytes	2	2	0	0	0
Mononuclear Cells ab	2	0	0	0	0
Eosinophils	4	0	0	0	0
Basophils	4	0	0	0	0
Calcium	4	0	0	0	0
Lipase	4	0	0	0	0
Amylase	3	1	0	0	0
Sodium	4	0	0	0	0
Potassium	3	1	0	0	0
Chloride	3	1	0	0	0
Carbon Dioxide	2	2	0	0	0
Urea/Nitrogen	3	1	0	0	0
Creatinine	3	1	0	0	0
Total Bilirubin	4	0	0	0	0
ALT	2	2	0	0	0
AST	4	0	0	0	0
Alkaline Phosphatase	4	0	0	0	0

a - These measurements were not taken for Arizona Patients.

Administration of an effective amount of the claimed compositions, either as a prophylactic dietary supplement or a pharmaceutical, is within the teachings of the invention. The term "effective amount" refers to an amount which alters the expression of certain types of proteolytic activities. Such an amount can be determined by those of skill in the art in accordance with known methods. For example, based on information determined in the accompanying Example, the preferred dose is 300-3000mg/day, more preferably 400-1000 mg/day, most preferably about 800 mg/day. However, doses in the range of 200-4000 mg/day are effective in humans ( $50-100 \mu\text{g/ml} \times 4000 \text{ ml}$  (average blood volume in man) = 200-400 mg BBIC; (100-400 CI units of BBIC is equivalent to 1000-4000 mg BBIC, as described in



Kennedy, *Prevent. Med.* 22:796-811, pp. 797 (1993D)). Optimal dosage may vary by body weight.

Further, based on data from the published literature, doses of purified BBI as low as 1.3 mg/day (in rats) and more than 150 mg/day are effective in animal models of carcinogenesis (St. Clair *et al.*, *Cancer Res.* 50:580-586 (1990); Kennedy, 1995C; van Hofe *et al.*, *Carcinogenesis* 12:2147-2150 (1991)). Doses lower than 1 mg/day to rats are likely to be effective as well (Kennedy, *J. Cell. Biochem.* 22:188-194 (1995D)), with doses of as little as 0.001 .mu.g/ml showing activity *in vitro* to suppress the malignant transformation of irradiated cells (Yavelow *et al.*, 1985). Such *in vitro* results would suggest that doses considerably lower than human doses of 2000 mg (2000 mg BBIC = 200 CI units) BBIC per day might be effective in the treatment of inflammation.

Compositions of the present invention may be administered parenterally, rectally, topically, transdermally or orally. However, the preferred administration is oral. Administration as either a prophylactic dietary supplement or as a pharmaceutical are contemplated. Published studies have shown that BBI is effective following a variety of routes of administration, including oral dosing (Kennedy, 1995C; Evans *et al.*, *Radiat. Res.* 132:259-262 (1992)). Examples of pharmaceutical or prophylactic dietary supplement formulations include, but are not limited to, syrups, suspensions, emulsions, tablets, capsules, lozenges and mouthwashes.

One embodiment of the invention is a liquid formulation comprising a suspension or solution of the composition in a pharmaceutically acceptable liquid carrier. Suitable liquid carriers include, but are not limited to, ethanol, glycerin, non-aqueous solvents such as polyethylene glycols, oils or water with a suspending agent, preservatives, flavorings or coloring agents, or any suitable combination thereof.

In another embodiment, a composition in the form of a tablet is prepared using any suitable pharmaceutical carrier routinely used for preparing solid formulations. Examples of such carriers include, but are not limited to, magnesium stearate, starch, lactose, sucrose and cellulose. Pharmaceutically acceptable fillers and supplements may also be added.

Compositions in the form of capsules are prepared using routine encapsulating procedure. For example, pellets, granules or powder containing a composition of the instant invention can be prepared using standard carriers and then filled into a hard gelatin capsule.



Alternately, a dispersion or suspension can be prepared using any suitable pharmaceutical carrier(s), and the dispersion or suspension is then filled into a soft gelatin capsule. Suitable pharmaceutical carriers include, but are not limited to, aqueous gums, cellulose, silicates and oils.

5 In yet another embodiment, a composition for parenteral administration is formulated as a solution or suspension. This solution or suspension will generally include the composition of the instant invention in a sterile aqueous carrier or parenterally acceptable oil. Examples of parenterally acceptable oils include, but are not limited to, polyethylene glycol, polyvinyl pyrrolidone, lecithin, arachis oils and sesame oil. Alternatively, the solution can be lyophilized  
10 and then reconstituted with a suitable solvent just prior to administration.

In addition, the BBI/BBIC composition is administered to the patient in conjunction with other drugs, medicaments, treatments or therapies to achieve reduction, suppression, inhibition or elimination of inflammation in the patient suffering from an inflammatory autoimmune disease, particularly a neuroinflammatory disease.

15 The invention is further described by example. The examples, however, are provided for purposes of illustration to those skilled in the art, and are not intended to be limiting. Moreover, the examples are not to be construed as limiting the scope of the appended claims. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a  
20 result of the teaching provided herein.

## EXAMPLES

Although the certain of the disclosed examples were conducted using recognized animal models to allow for the detailed *in vivo* evaluation of the therapy, the findings are directly  
25 applicable to human neuroinflammatory autoimmune disease, particularly MS, Guillain Barre Syndrome and rheumatoid arthritis, and provide the foundation for additional studies in to the nature of the inflammatory response in humans.

### Example 1 - BBIC inhibits Experimental Allergic Neuritis (EAN) in rats.

30 EAN was induced in seven week old Lewis rats using a synthetic peptide (SP26) corresponding to amino acid residues 53-78 of bovine myelin P2 protein. A solution of peptide



in normal saline was emulsified with Complete Freund's Adjuvant (CFA) in a 1:1 proportion and injected into the hind footpads. A total of 150 µg peptide and 100 µl of CFA was injected into each rat. A total of ten rats were injected. Of the ten animals, five (5) of the rats were also given 200 mg of BBIC in normal saline orally the first five days of every week. The remaining five (5) control rats were given saline only, without BBIC.

The rats were observed and clinically scored daily according to the following criteria, with half points used for intermediate signs: 0=normal; 1=flaccid tail; 2=flaccid tail, plus inability to spread toes; 3=paraplegia; 4=quadriplegia; 5=death (Rostami *et al.*, 1990).

The results of this study were striking. The BBIC-treated rats showed clinical signs of EAN for only 12 days; whereas the control rats showed clinical signs of EAN for 27 days, and remained symptomatic at the conclusion of the experiment. In both the control animals and the BBIC-treated animals, the peak in disease severity was manifested on day 17 post immunization (pi.). At the peak of the disease, the clinical scores of the 5 control rats averaged about 3 (paraplegia, hindlimb paralysis), while the scores of the 5 BBIC-treated rats averaged less than 1 (flaccid tail). In other words, the animals given BBIC were walking on all four limbs, while the control animals were dragging their paralyzed hindlimbs.

#### Example 2. Inhibition of EAN by Soybean Bowman Birk Protease Inhibitor.

To further understand the unique properties of BBIC as therapeutic treatment that can substantially decrease or even completely block the clinical signs of EAN, the following experiment was conducted. The inhibition was shown to be associated with the induction of apoptosis of CD4<sup>+</sup> T lymphocytes, resulting in the absence of effector cells in the EAN lesions, followed by the subsequent amelioration of the disease.

#### *Materials and Methods*

*Animals.* Female Lewis rats (n=12, Charles River, Raleigh, NC), weighing 140-150 (g), were immunized with 150 µg of SP26 as reported previously (Rostami *et al.*, 1990). Rats were observed daily for body weight change and clinical signs of EAN, which were scored as in Example 1.

*BBIC treatment.* Treatment was started on day 1 post immunization (pi.) and terminated at day 20 pi. For each experiment a group of rats (n=6) was provided with BBIC (Pharmacia Leo Therapeutics, Helsinborg, Sweden). Rats were giving 500 mg/ml of BBIC, in



drinking water, every day on a regular basis. PBS-treated EAN rats were used as a control as described in Example 1. Following BBIC treatment, disease severity was scored according to the above-described scale (mean  $\pm$ SD; n=6) for each group at each time point.

*Tissue preparation.* Rats were sacrificed at days 0, 7, 10, 13, 17, 20, and 24 pi. Quickly harvested cauda equina (CE), from which the fragments of spinal cords had been carefully excluded, and sciatic nerves (SN) were rapidly frozen in isopentane precooled in liquid nitrogen, and stored at -70° C.

*Histopathological evaluation.* Sections of CE and SN obtained from treated and untreated rats (PBS control) were stained with hematoxylin/eosin to identify mononuclear infiltrates. Areas of demyelination were identified in sections stained with luxol fast blue. Sections were evaluated and graded using the following scale: 0 = normal perivascular area; 1 = mild cellular infiltrate adjacent to a vessel; 2 = cellular infiltrate plus demyelinated fibers adjacent to a blood vessel and in the immediate surroundings; 3 = cellular infiltrate and demyelinated fibers around the vessels, confluence with infiltrates from more distant vessels.

*Cell preparation.* Rats were sacrificed at day 13-pi., and perfused with 150 ml of ice-cooled PBS via left ventricle. Spleens were removed and pooled in ice-cooled RPMI-1640 (Life Technologies, Gaithersburg, MD) containing 2% fetal calf serum (FCS) (Sigma, St. Louis, MO). A single cell suspension was obtained from spleens by pressing them through a 70  $\mu$ m Falcon cell strainer 2350 (Becton Dickinson, San Jose, CA). Erythrocytes in suspension were lysed by Tris-buffered ammonium chloride. Thioglycollate-elicited peritoneal macrophages ("elicited macrophages") were isolated 4 days later, after intraperitoneal stimulation of the rats with 20 ml of thioglycollate medium, and suspended in complete RPMI 1640 supplemented with 10% FBS, at  $10^6$  cells/ml. Elicited macrophages were comprised of 85-90% typical mononuclear phagocytes. All isolated cells were counted, after which cell viability was determined by trypan blue exclusion and found to be > 95%.

*Proliferation assay.* The proliferation response of the splenocytes was examined by  $^3$ H-thymidine incorporation. Briefly, 200  $\mu$ l of splenocyte suspension ( $10^6$  cells/ml) were incubated in 96-well polystyrene microtiter plates (Nunc, Roskilde, Denmark) at 37° C in a 5% CO<sub>2</sub> incubator, with or without SP26 (50  $\mu$ g/ml) in the presence or absence of BBIC (500  $\mu$ g/ml). After 60 hours, cells were pulsed with by  $^3$ H-thymidine (1  $\mu$ Ci/well; Amersham) for



12 hours. Cells were harvested and  $^3\text{H}$ -thymidine incorporation was measured in a liquid  $\beta$ -scintillation counter.

*Immunostaining and FACS analysis.* Fluorescein (FITC)-labeled anti-rat CD3 (mouse IgG<sub>3</sub>, G4.18 clone), CD4 (mouse IgG<sub>2a</sub>, OX-35), CD8 (mouse IgG<sub>1</sub>, OX-8), and rat  
5 macrophages (anti-ED1 mouse IgG<sub>1</sub>, k: 1C7) monoclonal antibodies, and phycoerythrin (PE)-labeled anti-rat CD3, CD4, CD8, CD25 (mouse IgG<sub>1</sub>, OX-39) and NKR-P1A (mouse IgG<sub>1</sub>, 10/78) monoclonal antibodies were purchased from PharMingen, San Diego, CA. FITC-labeled anti-rat CD11b (mouse IgG<sub>2a</sub>, OX-42) and CD25 monoclonal antibodies, PE-labeled anti-rat CD11b and B cells (mouse IgG<sub>2a</sub>, RLN-9D3) monoclonal antibodies were purchased  
10 from Caltag Laboratories, Burlingame, CA. One million ( $10^6$ ) cells were stained in 5 ml FACS tubes by 1  $\mu\text{g}$  of each monoclonal antibody following standard protocol (PharMingen). Labeled cells were analyzed by FACScan (Becton Dickinson) operated by CellQuest software (Becton Dickinson).

*Detection of apoptosis.* Apoptosis was determined using Flurescein Apoptosis  
15 Detection System Kit (Promega, Madison, WI). At day 13 pi., splenocytes from normal or EAN rats were obtained and cultured with BBIC (500  $\mu\text{g}/\text{ml}$ ), SP26 (50  $\text{ng}/\text{ml}$ ), or combinations thereof, for 72 hours. Cell suspensions were fixed with 4% paraformaldehyde for 30 minutes at room temperature and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice. TUNEL reaction mixture (50  $\mu\text{l}$ ) was added to samples  
20 incubated in a humidified chamber for 60 minutes at 37° C in the dark. Cell suspensions were then incubated with propidium iodide (PI) (Sigma). The cells were analyzed as above, by FACScan operated by CellQuest software.

*Cytokine enzyme-linked immunosorbent assays (ELISAs).* IL4 and IFN $\gamma$  were assayed by ELISAs developed by the researcher's laboratory. The lower and upper ranges for this  
25 assay were from 0.039 to 2.5  $\text{ng}/\text{ml}$ , respectively. IFN $\gamma$  determinations covered a range from 0.02 to 2.0  $\text{ng}/\text{ml}$ . An IL-10 assay was performed in accordance with manufacturer's recommendations (Endogen, Inc. Woburn, MA). Data were expressed as the mean cytokine concentration per ml from replicate determinations  $\pm$ SEM.

*Measurement of nitrite.* Nitrite (NO) was assayed by measuring the end product nitrite,  
30 which was determined by a colorimeter assay based on the Griess reaction. Aliquots of cell culture supernatants (100  $\mu\text{l}$ ) were mixed with 100  $\mu\text{l}$  of Greiss reagent at room temperature for



10 minutes. The absorbance was measured at 540 nm in an automated plate reader. The concentration of nitrite was determined by reference to a standard curve of sodium nitrite (Sigma).

*Statistics.* Data were analyzed by using the Student's t-test, wherein  $P < 0.05$  indicated that the value of the test sample was significantly different from that of the control level.

## Results

### *Effects of BBIC on the clinical course and histological signs of EAN.*

*BBIC treatment in vivo ameliorates the clinical course of active EAN.* All animals immunized with SP26 developed flaccid, ascending paralysis starting from the tail at day 11-12 pi. The disease reached its peak at day 16 pi., then recovery started (FIG. 1, control). Rats recovered clinically by about day 30 pi. with mild sequelae. The mean duration of the disease was  $23 \pm 2.6$  days, with a mean score of  $3.0 \pm 0.25$ . Rats immunized with CFA plus PBS without SP26 showed no clinical signs of disease (data not shown).

To determine whether oral BBIC could modify the clinical course of active EAN, the treatment was started on day 1 pi., and terminated at day 20 pi. (FIG. 1, BBIC). Rats (groups of 6) were given 500 mg/ml of BBIC, in drinking water, every day on a regular basis. Matched rats that received only PBS were used as a control. Following BBIC treatment, disease severity was scored according to the above-described scale (mean  $\pm$ SD;  $n=6$  for each group at each time point).

As shown in FIG. 1, the data indicated that treatment with BBIC dramatically decreased clinical severity and pathologic expression of EAN in Lewis rats injected with the neuritogenic SP26 peptide when compared to that in animals fed the PBS diluent without BBIC. There was also significantly reduced inflammatory cell accumulation in the peripheral nerve tissue of BBIC-treated animals. Some evidence also suggested increased frequency of lymphocyte apoptosis in BBIC-treated animals (Fig. 2)

*BBIC treatment in vitro ameliorates clinical course of passive EAN.* To determine whether BBIC could modify the clinical course of passive EAN, the disease was induced by the adoptive transfer of  $10^7$  SP26-specific splenocytes, treated or untreated by BBIC *in vitro* for 72 hours before transfer. Following transfer, disease severity was scored according to the above-described scale (mean  $\pm$ SD;  $n=6$  for each group at each time point). On day 15 pi passive



EAN/BBIC-untreated and passive EAN/BBIC-pretreated animals were sacrificed and staining was performed.

As shown in FIG. 2, treatment by BBIC *in vitro*, but not *in vivo*, following cell transfer, significantly decreased clinical severity (inflammation) of passive EAN ( $P < 0.05$ ). A large number of cell infiltrates were observed in sections of CE and SN isolated either from EAN PBS- or BBIC-*in vivo*-treated animals (data not shown).

*Potential pathways involved in BBIC action.*

(i) *Inhibition of antigen-specific proliferation by BBIC in vitro.* As shown in FIG. 3, when splenocytes isolated from EAN animals (day 13 pi.) were incubated with or without SP26 in the presence or absence of BBIC, cellular proliferation was significantly enhanced in the presence of SP26 (significantly more  $^3\text{H}$ -thymidine was incorporated). By comparison, the proliferation of splenocytes incubated in culture media alone (without SP26) was not augmented. However, when the splenocytes were cultured with BBIC, cells proliferation was significantly decreased in the presence of SP26, and not affected in the resting cell population.

(ii) *Effects of the BBIC treatment on expression of CD3, CD4, CD8, CD11b, and ED-1.*

To characterize the influence of BBIC on T cell, B cell, NK cell and macrophage populations, cell debris was excluded from the data using forward and side light scatter plots (FIGs. 4 and 5), and cells were analyzed for  $\text{CD3}^+$ ,  $\text{CD4}^+$ , and  $\text{CD8}^+$  (T cells) (FIG. 4);  $\text{CD11b}^+$  and  $\text{ED-1}^+$  (macrophages) (FIG. 5);  $\text{NKR-P1A}^+$  (NK cells); and pan-B markers. The data in FIG. 4 and FIG. 5 are representative of 6 different experiments.

The numbers in the figures refer to the mean fluorescence intensity (MFI), and the percentage of positive cells, plus they show that BBIC (FIG. 4, panels D, H, and L) down-regulated expression of CD3 (FIG. 4, panel D) and CD4 (FIG. 4, panel H) relative to expression by the cells analyzed after incubation in culture media (FIG. 4, panels B and F), or after treatment with SP26 alone (FIG. 4, panels C and G).

BBIC had no effect on CD8 (FIG. 4, panel L) or CD 11b (FIG. 5, panel D) expression, but increased the expression of ED-1 from 3% (FIG. 5, panel F) to 14% (FIG. 5, panel H). Thus, it appears that BBIC treatment stimulated the macrophage population. The expression of NK and B cell markers, analyzed in the presence or absence of SP26, treated or untreated by BBIC, had no changes and was almost negligible (data not shown).



(iii) *Influence of BBIC treatment on IFN $\gamma$ , IL-10, IL-4, and NO release.* Previous data indicated that macrophages can capture, process, and present antigens to T cells, and produce cytokines and mediators to participate in immune responses. To further elucidate the mechanisms involved in the suppression of EAN after BBIC administration, either elicited  
 5 macrophages or splenocytes were obtained from PBS and BBIC -treated EAN rats at day 13 pi., and their activities were evaluated. Compared with PBS-treated rats, cells isolated from BBIC-treated rats showed higher secreted levels of IFN $\gamma$ , both spontaneously and upon stimulation with SP26 (data not shown). Treatment by BBIC further augmented IFN $\gamma$  release (FIG. 6A). At the same time, it was found that IL-10 release was not increased, either in the  
 10 presence or absence of SP26, treated or untreated by BBIC (FIG. 6B). In addition, IL-4 was not detected in the culture fluids, under any of the conditions used (data not shown).

When macrophages were cultured with BBIC (500  $\mu$ g/ml) for 4 hours (FIG. 7A) or 24 hours (FIG. 7B), nitrite production was dramatically augmented, suggesting that macrophage activation induced by BBIC treatment may increase IFN $\gamma$  release, and consequently promote  
 15 nitrite production by these cells.

(iv) *Induction of differential cell apoptosis by BBIC in vitro.* It has been reported that nitrite production is frequently associated with impaired T cell responses due to apoptosis and macrophage activation. Since the present results indicated that macrophage function are elevated by BBIC treatment, the apoptotic response was characterized for spleen cells isolated  
 20 from EAN rats. On day 13 pi., splenocytes were obtained and incubated for 72 hours, with or without SP26, in the presence or absence of BBIC. Then, gated by size (FIG. 8, panel A), cells were analyzed by flow cytometry for an apoptotic response.

In the absence of SP26 stimulation, the level of apoptosis was higher in the population of larger cells (27.5% (FIG. 8, panel B)) compared to relatively smaller cells (12.5% (FIG. 8, panel F)). This finding was explained by extrinsic effects, such as Fas/FasL-mediated  
 25 apoptosis or cellular response to SP26 immunization. However, it was determined that upon *in vitro* stimulation with SP26, the R1 population (larger cells) was not affected after 72 hours of incubation in the presence or absence of BBIC (FIG. 8, panels C to E, respectively). In a co-culture of the R2 population (smaller cells) with a combination of SP26 and BBIC (FIG. 8, panel I), a much higher level of apoptosis (32.5%) was observed. This corresponded to the  
 30



previously shown decrease in antigen-specific proliferation of splenocytes (FIG. 3), and to changes in CD3<sup>+</sup>, CD4<sup>+</sup> expression (FIG. 4, panel D and panel K, respectively).

In sum, the immunomodulatory effects of BBIC on this experimental model of disease were associated with marked inhibition of antigen-specific proliferation and a decrease in CD3- and CD4-positive T cell subpopulations. Furthermore, treatment with BBIC substantially increased the number of macrophages, stimulated IFN- $\gamma$  and nitrite release, and resulted in lymphocyte apoptosis. Accordingly, the apoptosis of lymphocytes induced by BBIC in the peripheral nervous system and lymphoid organs are directly associated with the absence of inflammatory cells in the EAN lesions and amelioration of the disease.

Example 3. Randomized, double-blind, placebo-controlled, study of safety, tolerance and clinical effects of the oral BBIC in Multiple Sclerosis

In early studies, orally administered BBIC was seen to inhibit both the clinical and pathologic manifestations of EAE in Lewis rats inoculated with myelin basic protein (MBP) in complete Freund's adjuvant (FIGs. 9, 10). In light of this effect on EAE, a recognized and accepted animal model for human MS, a 1-year pilot study was planned to determine that oral administration of BBIC in the dose specified, is well-tolerated by patients with active MS, and to obtain a preliminary impression of the efficacy of BBIC treatment in halting the progression of relapsing-remitting MS. The results of the study will be quantified and correlated with effects determined in laboratory assays and brain assays. Analyses of blood and urine and brain MRIs will occur before BBIC therapy begins, and periodically during and after the BBIC therapy period. In addition, the level of markers of inflammation in blood are assessed. Any potential side effects of the BBIC treatment is closely monitored

The clinical subjects consist of twenty individuals (ages 18-65) male and female, with well documented MS in a relapsing-remitting course are registered. Each patient must have exhibited at least one relapse during the year prior to initiation of the BBIC trial. These individuals are not receiving glatiramer or beta-interferon (INF- $\beta$ ), because of previously manifested side effects of these treatments or because the patient did not choose to receive glatiramer or INF- $\beta$ .

All MS patients considered for this phase I study must be followed in the MS center of the Hospital of the University of Pennsylvania Medical Center. Eligibility for enrollment is



based on independent examination by 2 staff physicians in the MS clinic using a standardized patient questionnaire and evaluation form. Informed Consent is obtained after a detailed discussion with the patient/family members about the project, including any potential risks of treatment.

5 All patient data is recorded and maintained on Standardized Case Report Forms (CRF) for subsequent data analysis and reporting. All data analysis is carried out by the (EMMES) consulting agency.

Patient exclusion criteria include:

- 10 1) Current pregnancy or lactation (baseline pregnancy test if there is any question concerning status). Also, females of child-bearing potential who are not practicing an acceptable method of contraception/abstinence, barrier method, oral contraception.
- 2) Age less than 18 or greater than 65.
- 3) MS in remission or quiescent (DSS at baseline <3.0) or extremely severe (DSS>6.5)
- 15 4) Beta interferon, glatiramer or corticosteroid therapy within 3 months prior to baseline. Immunosuppressive treatment.
- 5) Primary MS brain stem/visual symptoms only without spinal cord manifestations.
- 6) Medical conditions which may confound evaluation of BBIC treatment in MS (e.g. ALS, vasculitis, syphilis, HIV or HTLV-1 myelopathy, active thyroid disease, nutritional deficiencies, life-threatening diseases).
- 20 7) Any prior diagnosis of pancreatitis, pancreatic carcinoma, pancreatic adenoma, obstruction of pancreatic ducts, or amyloidosis.
- 8) Allergy, or prior adverse reaction to soybeans.
- 9) Patient with high levels of BBI activity in the diet (as determined from the food frequency questionnaire, or direct measurement of BBI metabolites in blood or urine).
- 25

The BBIC used in the study contains proteins and carbohydrates, with essentially no fat. BBIC contains 100 mg/g CI activity, and 40 mg/g trypsin inhibitory activity. The CI activity in BBIC is stable over at least a 1 1/2 year period, as is the ability of BBIC to inhibit transformation *in vitro*. BBIC is manufactured by Central Soya, Inc. (Ft. Wayne, IN) and the tablet formation prepared by Murty Pharmaceuticals, Inc. (Lexington, KY).

35 *Therapy.* BBIC tablets or placebo is administered orally on a daily basis, for 12 months. The BBIC of 800 CI units per day is divided into two fractions, to be taken with food two times per day in the morning and in the evening. Of the total of 20 patients in the trial, 15 receive BBIC (800 CI units/day) and 5 receive placebo in a randomized, double-blinded manner. Medications (BBIC and placebo) are dispensed by a pharmacist, who randomly



assigns medication with only a coded label. Only the pharmacist knows the treatment groups to which the patients have been assigned. The pharmacist is not involved in other aspects of the trial, and will not make the code available until after all of the data from the trial have been recorded and analyzed.

5        *Evaluation methods.* Each patient is treated as an out-patient. Baseline vital signs and a physical examination are carried out before initiation of the therapeutic regimen to assess any possible contra-indications to the treatment.

Lab safety assessments:

a) Baseline measurements

Blood Chemistries

-Glucose	-ALT(SGPT)
-Calcium	-Total Protein
-Urea	-Albumin
-Creatinine	-Cholesterol
Total Bilirubin	-Alkaline Phosphatase
-AST (SGOT)	-Phosphorus

Electrolytes

-Sodium	-Potassium
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Hematology

-Hemoglobin	-Bands
-Hematocrit	-Lymphocytes
-Red Blood Cells (RBC)	-Monocytes
-RBC Morphology	-Eosinophils
-White Blood Cells	-Basophils
-Neutrophils	-Platelets

Urinalysis

-Protein	-Glucose
-pH	-Blood

Serum Beta hCG, Qualitative

*Sequential safety studies.*

- (1) Baseline and every 3 months - CBC, urinalysis, liver function tests, chemistry panel, sedimentation rate.
- (2) Baseline and at 6 and 12 months - chest x-ray.
- (3) Baseline and after one (1) year - levels of anti-mouse Ig antibodies.
- (4) Baseline and after one (1) year - serum levels and complement C4 and C3.

*Clinical assessment of MS status.*

Baseline and every 3 months:

- Physical Examination-(including Vital Signs) -Medical History
- Historical/Concomitant Medications
- Medications
- ECG



EDSS Evaluation

- MSQLI
- 9-hole peg test
- PASAT (Cognitive function test)
- Timed, 25 meter ambulation test
- Contrast letter acuity testing with Sloan low-contrast letter charts
- Brain MRI, with gadolinium injection

All adverse experiences (ADR) during the pilot study, whether or not of immediate relationship to BBIC administration, must be recorded on the Toxicities/Medical Problems of the Case Report Form.

*Inflammation Studies.* Evaluation of general CMI. It is generally agreed that DTH skin testing is the most cost-effective method to assess *in vivo* CMI (Kennedy, 1998). Extensive clinical experience indicates that 90% of healthy adults exhibit DTH reactions to one or more of a panel of 5 antigens. DTH skin testing will be conducted before treatment and at 3 month interval thereafter by practitioners who are highly experienced in the technique, with excellent interrater reliability (Kennedy *et al*, 1993B). Five common recall antigens are used: mumps (Connaught; 40 complement-fixing units per ml); tetanus toxoid (Wyeth; 10 Loeffler units/ml); *Candida albicans* (Greer; 500 protein nitrogen units per ml); purified protein derivative (Connaught; 5 TU), and *Trichophyton* sp. (Hollister; 1:30). In quality control studies using freshly diluted reagents, which were used well within the expiration period of the stock solutions, patterns of responses are consistent over a period of years. Repeat testing in healthy volunteers induces similar responses. A reduction of >50% in the diameter of induration at 48 hours would be evidence of significant inhibition of DTH.

To study inflammatory reactivity CRP is measured in serum samples by an inhibition enzyme-linked immunoassay (ELISA). Alpha I -antichymotrypsin (ACT) levels in serum samples are assayed using an inhibition ELISA. Purified human ACT and anti-human ACT antibodies are commercially available (Calbiochem). Normal serum ACT average about 420 mg/l. Since serum MMP-9 levels are reportedly increased shortly before MS exacerbations (Hartung *et al.*, 2000), the effect of BBIC administration on MMP-9 levels is an important surrogate measurement of the inhibitory effect of BBIC in the enzymatic cascade. Serum MMP-9 levels will be assessed by an immunoassay, as described by Gijbels *et al.*, 1992.



Functional determinations are measured and quantified according to the following standardizing scales to determine initial capability of the patient and to measure changes resulting from treatment.

Functional System Scale Studies (FS)

5 Pyramidal functions:

- 0 = Normal
- 1 = Abnormal signs without disability
- 2 = Minimal disability
- 3 = Mild or moderate paraparesis, herniparesis, or severe monoparesis
- 10 4 = Marked paraparesis or herniparesis, or moderate quadriparesis or monoplegia
- 5 = Paraplegia, herniplegia, or marked q4adriparesis
- 6 = Quadriplegia

Sensory Functions:

- 15 0 = Normal
- 1 = Vibration or figure writing decrease 1 or 2 limbs
- 2 = Mild decrease in touch or pain or position sensation and/or moderate decrease in 1 or 2 limbs; vibratory decrease alone in 3 or 4 limbs
- 3 = Moderate decrease in touch or pain or position sensation and/or essentially lost vibration in 1 or 2 limbs; or mild decrease in touch or pain and/or moderate decrease in proprioceptive tests in 3 or 4 limbs
- 20 4 = Marked decrease in touch or pain or proprioception alone or combined in 1 or 2 limbs; or moderate decrease in touch or pain and/or severe proprioceptive loss in more than 2 limbs
- 5 = Loss of sensation in 1 or 2 limbs; or moderate decrease in touch or pain and/or loss of proprioception below the head
- 25 6 = Sensation lost below the head

Cerebellar functions:

- 0 = Normal
- 30 1 = Abnormal signs without disability
- 2 = Mild ataxia
- 3 = Severe ataxia in all limbs
- 4 = Unable to perform coordinated movements

35 Bowel and Bladder Functions:

- 0 = Normal
- 1 = Mild urinary hesitancy, urgency, or retention
- 2 = Moderate hesitancy, urgency, retention of bowel or bladder, or rare urinary incontinence
- 3 = Frequent urinary incontinence
- 40 4 = In. need of almost constant catheterization, but with adequate bowel function
- 5 = Loss of bladder function
- 6 = Loss of bladder and bowel function



Brain Stem Functions:

0 = Normal

1 = Signs only

2 = Moderate nystagmus or other mild disability

5 3 = Severe, marked extraocular weakness or moderate disability of other cranial nerves

4 = Marked dysarthria or other marked disability

5 = Inability to swallow or speak

Mental Functions:

10 0 = Normal

1 = Mood alteration only

2 = Mild decrease in mentation

3 = Moderate decrease in mentation

4 = Marked decrease in mentation

15 5 = Dementia and/or chronic alertness

Visual Functions:

0 = Normal

1 = Acuity better than 20/30 in the worse eye

20 2 = Acuity between 20/30 and 20/59 in worse eye

3 = Acuity between 20/60 and 20/99 in worse eye

4 = Acuity between 10/100 and 20/200 in worse eye Grade 3 plus better eye 20/60 or less

5 = Acuity 20/200 or less in worse eye or Grade 4 plus better eye 20/60 or less

6 = Grade 5 plus better eye 20/60 or less

Other Functions:

0 = Normal

1 = Any other findings (specify below)

30 Ambulatory determinations are measured and quantified according to the following standardizing Ambulation Index scales to determine initial capability of the patient and to measure changes resulting from treatment.

Ambulation Index

35 0 = Asymptomatic; fully active.

1 = Walks normally but reports fatigue which interferes with athletic or other demanding activities.

2 = Abnormal gait or episodic imbalance; gait disorder is noticeable to family and friends. Able to walk 25 feet in 10 seconds or less.

40 3 = Walks independently; able to walk 25 feet in 20 seconds or less.

4 = Requires unilateral support (cane, single crutch) to walk; uses support more than 80% of the time. Walks 25 feet in 20 seconds or less.

5 = Requires bilateral support (cane, crutches, walker) and walks 25 feet in 20 seconds or less; or, requires unilateral support but walks 25 feet in greater than 20 seconds.



- 6 = Requires bilateral support and walks 25 feet in greater than 20 seconds. May use wheelchair on occasion.\*
- 7 = Walking limited to several steps with bilateral support; unable to walk 25 feet. May use wheelchair for most activities.
- 5 8 = Restricted to wheelchair; able to transfer independently.
- 9 = Restricted to wheelchair; unable to transfer independently.

\*The use of a wheelchair may be determined by a patient's lifestyle and motivation. It is expected that patients in grade 7 will use a wheelchair more frequently than patients in grades 5 or 6. Assignment of a grade, however, in the 5-7 range is determined by the ability of a patient to walk a given distance, and not by the extent to which a patient uses a wheelchair.

To define the functional and ambulatory determinations, the Kurtzke Expanded Disability Status Scale (EDSS) in Multiple Sclerosis follows. Note: EDSS steps below 5 refer to patients who are fully ambulatory, and the precise step is defined by the Functional System (FS) score(s). EDSS steps from 5 up are defined by ability to ambulate, and usual equivalents in FS scores are provided. A mental function grade of 1 does not enter into FS score for EDSS steps. EDSS should not change by 1.0 step unless there is a change in the same direction of at least one step in at least one FS.

#### Kurtzke Expanded Disability Status Scale (EDSS)

- 0 = Normal neurologic exam (all grade 0 in FS Cerebral grade I acceptable).
- 1.0 = No disability, minimal signs in one FS 9 (i.e., grade I excluding Cerebral grade 1).
- 1.5 = No disability, minimal signs in more than one FS (more than grade I excluding Cerebral grade 1).
- 2.0 = Minimal disability in one FS (one FS grade 2, others 0 or 1).
- 2.5 = Minimal disability in two FS (two FS grade 2, other 0 or 1).
- 3.0 = Moderate disability in one FS (one FS grade 3, other 0 or 1), or mild disability in three of four Fs (three/four FS grade 2, other 0 or 1) though fully ambulatory.
- 3.5 = Fully ambulatory but with moderate disability in one FS (one grade 3 and one or two FS grade 2; or two FS grade 3; or five FS grade 2 (others 0 or 1).
- 4.0 = Fully ambulatory without aid, self-sufficient, up and about some 12 hours a day despite relatively severe disability consisting of one FS grade 4 (other 0 or 1), or combinations or lesser grades exceeding limits of previous steps. Able to walk without aid or rest some 500 meters.
- 4.5 = Fully ambulatory. without aid, up and about much of the day, able to work a full day, may otherwise have some limitation of full activity or require minimal assistance; characterized by relatively severe disability, usually consisting of one FS grade 4 (others 0 or 1), or combinations or lesser grades exceeding limits of previous steps. Able to walk without aid or rest for some 300 meters.
- 5.0 = Ambulatory without aid or rest for about 200 meters; disability severe enough to impair full daily activities (e.g., to work a full day without special provisions).



Usual FS equivalents are one grade 5 alone, other 0 or 1; or combinations of lesser grades usually exceeding specifications for step 4.0.

- 5.5 = Ambulatory without aid or rest for about 100 meters; disability severe enough to preclude full daily activities. Usual FS equivalents are one grade 5 alone, others 0 or 1; or combinations of lesser grades usually exceeding those for step 4.0.
- 6.0 = Intermittent or unilateral constant assistance (cane, crutch, etc.) required to walk about 100 meters, with or without rest. Usual FS equivalents are combinations with more than two FS grade 3+.
- 6.5 = Constant bilateral assistance required to walk about 20 meters without resting. Usual FS equivalents are combinations with more than two FS grade 3+.
- 7.0 = Unable to walk beyond about 5 meters even with aid, essentially restricted to wheelchair; wheels self in standard wheelchair and transfers alone; up and about in w/c some 12 hours a day. Usual FS equivalents are combinations with more than one FS grade 4; very rarely pyramidal grade 5 alone.
- 7.5 = Unable to take more than a few steps; restricted to wheelchair, may need aid in transfer; wheels self but cannot carry on in standard wheelchair a full day; may require motorized wheelchair. Usual FS equivalents are combinations with more than one FS grade 4+.
- 8.0 = Essentially restricted to bed or chair or perambulated in wheelchair; may be out of bed itself much of the day; retains many self-care functions; generally has effective use of arms. Usual FS equivalents are combinations, generally grade 4+ in several systems.
- 8.5 = Essentially restricted to bed or chair or perambulated in wheelchair; may be out of bed much of the day; retains many self-care functions; generally has effective use of arms. Usual FS equivalents are combinations, generally grade 4+ in several systems.
- 8.5 = Essentially restricted to bed much of the day; has some effective use of arms; retains some self-care functions. Usual FS equivalents are combinations, generally 4+ in several systems.
- 9.0 = Helpless bed patient; can communicate and eat. Usual FS equivalents are combinations, mostly grade 4+.
- 9.5 = Totally helpless bed patient; unable to communicate effectively or eat/swallow. Usual FS equivalents are combinations, almost all grade 4+.
- 10.0 = Death due to MS.

Cognitive Function is measured and standardized initially and throughout treatment by the Paced Auditory Serial Addition Test (PASAT), which provides a measure of sustained attention and information processing speed. It was originally devised by Gronwall and colleagues (Gronwall, 1977; Gronwall & Wrightson, 1974, herein incorporated by reference) to measure the acute effects of minor head injury. The test requires the use of a cassette tape recorder and a prerecorded tape, which may be purchased with the Brief, Repeatable Battery. Two alternate forms of this test have been developed, although practice effects should not be a problem with this test.



Manual dexterity is measured and quantified according to the following standardized 9-Hole Peg Test to determine initial capability of the patient and to measure changes resulting from treatment.

## 5 9-Hole Peg Test

Pegboard is centered in front of subject with pegs placed in the container next to the board on the same side as the hand being tested. The dominant hand is tested first. While the examiner briefly demonstrates the test, the patient is instructed to pick up the pegs one at a time, using the dominant hand only, and to put the pegs into the holes in any order until all of the holes are filled. Then the patient is to remove pegs one at a time, and return them to the container. The goal is to move as quickly as possible through the test. A practice test is given first, followed by the actual test, during which the patient is told to go faster.

The stopwatch is started by the examiner as soon as the subject touches the first peg, and is stopped when the last peg hits the container, and the time is recorded in seconds. Then the container is placed on the opposite side of the pegboard and the patient's other hand is tested. There are two trials per hand. Maximum time to complete one trial is 5 minutes (300 seconds). For patients with upper extremity tremors or truncal ataxias, stabilization of elbows on the table surface may be used.

Following completion of the clinical trial, the method of using BBI/BBIC defined herein, is applied to treat patients with chronic inflammatory autoimmune diseases, such as rheumatoid arthritis, particularly those patients with chronic neuroinflammatory autoimmune diseases, such as MS or GBS. The tests and standards provided herein provide measures for determining levels of improvement in the patient as the inflammation is reduced, suppressed, alleviated or eliminated as a result of the BBI/BBIC treatment.

Each and every patent, patent application and publication that is cited in the foregoing specification is herein incorporated by reference in its entirety.

While the foregoing specification has been described with regard to certain preferred embodiments, and many details have been set forth for the purpose of illustration, it will be apparent to those skilled in the art that the invention may be subject to various modifications and additional embodiments, and that certain of the details described herein can be varied considerably without departing from the spirit and scope of the invention. Such modifications,



equivalent variations and additional embodiments are also intended to fall within the scope of the appended claims.

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